DB-ALM Protocol n° 158 : human Cell Line Activation Test (h-CLAT)

Skin Sensitisation & Allergic Contact Dermatitis

The h-CLAT is an *in vitro* method for measuring the expression of CD86 and CD54 protein markers on the surface of the human monocytic leukemia cell line, THP-1, used as a surrogate for human dendritic cells (DC), following controlled exposure to test chemicals (Ashikaga et al., 2006, 2010). The activation process in which DC change from antigen processing to antigen presenting cells is considered a key event in the acquisition of skin sensitisation. This activation process involves the modulation of the expression of cell surface phenotypic markers, those most commonly reported being CD54, CD80, CD86 and major histocompatibility complex [MHC] class II (Galvao dos Santos et al., 2009). Therefore, information from the h-CLAT method is considered relevant for the assessment of the skin sensitisation potential of chemicals.

Résumé

The purpose of the test method is to contribute to the identification of skin sensitisers and non-sensitisers by providing information on the chemical-induced expression of cell surface markers associated with the activation of dendritic cells (DC). The activation process through which DC change from antigen processing to antigen presenting cells addresses the third key event of the skin sensitisation Adverse Outcome Pathway (AOP; OECD, 2012).

Experimental Description

Endpoint and Endpoint Measurement:

MODULATION OF THE EXPRESSION OF CELL SURFACE PHENOTYPIC BIOMARKERS (CD86 AND CD54): quantified by flow cytometric analysis following 24 hour exposure of the THP-1 cell line to test chemicals.

Endpoint Value:

Relative Fluorescence Intensity (RFI) of CD86 and CD54 cell surface molecules.

Experimental System(s):

The test system is the THP-1 cell line purchased from an appropriate cell bank, such as ATCC, #TIB-202. THP-1 is an immortalized human monocytic leukemia cell line, used as a surrogate for DC.

Discussion

Given the complexity of the biological mechanisms underlying skin sensitisation, it is likely that a combination of mechanistically-based test methods within Integrative Approaches to Testing and Assessment (IATA) is needed to fully replace the animal tests currently used for satisfying regulatory requirements. Since the h-CLAT method addresses a key biological mechanism involved in the induction of skin sensitisation, it provides useful information within integrated approaches for skin sensitisation assessment to replace or reduce animal testing (Nukada et al., 2012, 2013 and Tsujita-Inoue et al., 2014).

Status

Known Laboratory Use:

Kao Corporation

Shiseido Co., Ltd.

BASF

Bioassay

Participation in Evaluation Studies:

The h-CLAT method was optimised in Cosmetics Europe (former COLIPA) coordinated ring trials (Sakaguchi et al., 2010) and in a Japanese ring study supported by the Japanese Ministry of Health, Labour and Welfare (MHLW) (Ashikaga et al., 2008).

Participation in Validation Studies:

The h-CLAT method was the subject of a EURL ECVAM validation study for assessment of transferability as well as within- and between-laboratory reproducibility, which showed the h-CLAT method to be transferable to suitably equipped laboratories that are proficient in cell culture techniques and flow cytometry. The results demonstrated an overall within-laboratory reproducibility of 80.0 % and a between-laboratory reproducibility of 79.2% (h-CLAT validation study report 2012). EURL ECVAM published the recommendation on the h-CLAT method on 02.03.2015 (EURL ECVAM, 2015).

Regulatory Acceptance:

The test method was adopted as **OECD Test Guideline No. 442E** in July 2016. TG 442E describes an *in vitro* procedure proposed for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (UN, 2013). An updated version of the OECD TG442E "In Vitro Skin Sensitisation: In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation" has been issued in October 2017 and further updated in June 2018 (OECD, 2018).

Proprietary and/or Confidentiality Issues

Intellectual property rights to the h-CLAT method are protected by law in Japan (Patent N° 4270702).

Anyone interested in the implementation of the method in Japan should contact Shiseido Co., ltd (see contact details on page 4).

Health and Safety Issues

General Precautions

General safety instructions should be followed and appropriate protective safety equipment worn at all times. Unknown and coded chemicals should be considered potential sensitising agents or toxins and must be handled with extreme care.

MSDS Information

Propidium Iodide (CAS 25535-16-4) is a known carcinogen. MDS Available at: **www.sigmaaldrich.com**

Abbreviations and Definitions

AOP : Adverse Outcome Pathway

ATCC: American Type Culture Collection

BSA: Bovine Albumine Fraction

CV75: Estimated concentration giving 75% cell viability

DB-ALM: EURL ECVAM DataBase service on Alternative Methods to animal experimentation

DCs : Dendritic cells

DMSO: Dimethyl Sulfoxyde

DNCB : 2,4-dinitrochlorobenzene

EC: Effective Concentrations

EC50: Effective concentration 50

EC200: Effective concentration 200

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FcR: Fc receptor

FITC: Fluorescein Isothiocyanate

FSC: Forward Scatter

h-CLAT: Human Cell Line Activation Test, also abbreviated as hCLAT

HSC: Highest Soluble Concentration

IATA: Integrative Approaches to Testing and Assessment

IgG1: Immunoglobulin G1

Kow: Octanol-Water Partition Coefficient

LA: Lactic acid

LCs : Langerhans cells

MFI : Mean Fluorescence Intensity

MHC : Major Histocompatibility Complex

MHLW: Ministry of Health, Labour and Welfare

MSDS: Material Safety Data Sheet

$NiSO_4$: Nickel sulphate

PBS: Phosphate buffered salts

PI: Propidium iodide

RFI: Relative Fluorescence Intensity

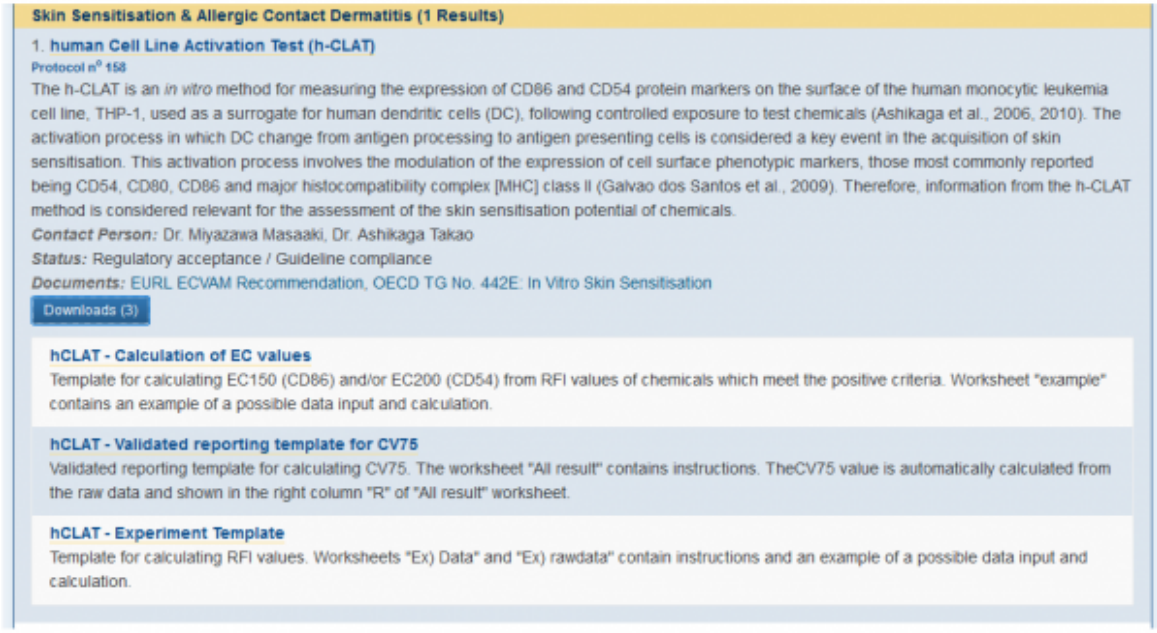
SSC: Side scatter

Last update: 23 July 2018

PROCEDURE DETAILS, 29 November 2012 (Version 7)

human Cell Line Activation Test (h-CLAT)
DB-ALM Protocol n° 158

The protocol is based on the Standard Operating Procedure (SOP) used in the validation study on the h-CLAT Method. Study templates are available from DB-ALM website. Go to the section related to the Protocol No. 158 and select *Related information* - **Downloads** (snapshot below).



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Materials and Preparations

Cell or Test System

The THP-1 cell line is an immortalized human monocytic leukemia cell line, used as a surrogate for DC. This cell line is available for purchase from appropriate cell banks, such as ATCC, #TIB-202.

Equipment

Fixed Equipment

Standard cell culture laboratory equipment including an incubator with CO_2 supply, biological safety cabinet and centrifuge.

Specific Equipment

Flow cytometer: FACSCalibur and FACSCanto (Becton Dickinson) or EPICS XL-MCL System II (Beckman Coulter) or equivalent.

Consumables

Impurities found in antibiotics or other materials as well as in disposable tubes or other disposables can affect the character of THP-1 cells. The quality of materials and disposables is to be carefully confirmed before use. Proven (or specified) reagents, tools, and disposables essential to proper conduct of the test are listed below:

- Culture flask or culture dish (non-treatment): The size of flasks and dishes for culture will vary per the volume of the cell suspension (e.g. Non-tissue culture treated flask 250 mL, BD Falcon, #353133)
- Sample tube: Polypropylene round tube 5 mL, BD Falcon, #352054, or 1.5 mL microtube, Eppendorf, #30121589
- 24-well flat-bottom plate (e.g. BD Falcon, #351147)
- 96-well flat-bottom plate (e.g. BD Falcon, #351172)
- 96-well round-bottom plate (e.g. BD Falcon, #353910)
- Volumetric flask (e.g. Asahi Glass, #72-088-504)
- Glass vial or tube (e.g. Asahi Glass, # 71-099-006)

Media, Reagents, Sera, others

For maintenance of THP-1 cells

- RPMI-1640: GIBCO, #22400-089 (containing 25 mM HEPES buffer and L-glutamine)
- Fetal bovine serum (FBS): GIBCO, #10099-141. FBS is to be inactivated by heating to 56˚C for 30 minutes and tested by verifying the reactivity of THP-1 cells
- 2-Mercaptoethanol: GIBCO, #21985-023
- Antibiotics (e.g. Penicillin-Streptomycin, GIBCO, #15140-122)

For chemical exposure

- Physiological saline: solution of 0.91 % (w/v) of NaCl - Otsuka Pharmaceutical or Sigma-Aldrich, #S8776. Referred to as “saline” throughout the protocol
- Dimethylsulfoxide (DMSO): Sigma-Aldrich, #154938-500mL

For flow cytometry

- Calibration beads (e.g. Calibrite beads3, Beckton and Dickinson (BD), #340486)
- Phosphate buffered salts Dulbecco’s formula without magnesium, calcium or phenol red (PBS): GIBCO, #14190-136, or Nisssui Pharmaceutical, #018-17854
- Bovine Albumin Fraction V powder (BSA): Calbiochem, #12660, or Wako Pure Chemical Industries, #5193
- Globulins Cohn fraction II, III, Human: SIGMA, #G2388-10G
- Propidium iodide (PI): SIGMA, #P4170-25MG

- Antibodies, specified as follows:
  - FITC-labelled mouse monoclonal anti-human CD86 antibody: BD-PharMingen, #555657 (Clone: Fun-1)
  - FITC-labelled mouse monoclonal anti-human CD54 antibody : DAKO, #F7143 (Clone: 6.5B5)
  - FITC labelled-mouse IgG1: DAKO, #X0927

Preparations

Media and Endpoint Assay Solutions

Culture medium:
RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (v/v), 0.05 mM 2-mercaptoethanol and appropriate antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) is used to culture the cells and during the assay. Medium with supplements must be stored at 4˚C and used within one month.

The culture medium must be warmed to room temperature just before use.

Medium for freezing cells:
Culture medium supplemented with 10% (v/v) sterile dimethylsulfoxide (DMSO).

Flow Cytometry reagents:

FACS buffer:
PBS with 0.1 % (w/v) BSA, should be prepared a day before use.

Blocking solution:
0.01 % (w/v) globulin solution in FACS buffer. A 1% solution is made by PBS a day before use and stored at 4˚C, and should be used within one week. The blocking solution is prepared by 1:100 dilution of 1 % solution with FACS buffer just before use.

PI solution:
12.5 µg/mL of propidium iodide in PBS, store at 4˚C in the dark (for example, hundredfold concentrated PI stock solution (1.25 mg/mL) can be prepared in advance).

Test Compounds

Solubility of each chemical in an appropriate solvent is evaluated and confirmed. (See section on **Test Material Exposure Procedures**, p.10)

Positive Control(s)

Reactivity check: 2,4-dinitrochlorobenzene (DNCB) and Nickel Sulfate ($NiSO_4$)

Test: DNCB

Negative Control(s)

Reactivity check: Lactic Acid (LA)

Test: A medium control sample and a solvent control sample for DMSO or other solvents are to be included. A solvent control sample for saline is not needed, however, because the medium control serves as its alternative.

Method

Test System Procurement

Stocks are to be prepared by the test lab from seed cells obtained from a reliable cell bank (e.g. ATCC, #TIB-202).

After thawing according to cell bank's instructions, expand by culturing within one month. Batches are then prepared by freezing aliquots of cells in freezing medium at 1 to 2 × 10^6 cells/mL. Cells from the stock are then used for routine testing.

Routine Culture Procedure

FOR CELL CULTURE

Thawing and freezing :
After obtaining seed cells from a reliable cell bank, thaw according to cell bank's instructions, and expand by culturing within one month.

When preparing stock for freezing, the cells are harvested, pelleted by centrifugation (e.g., approximately 250g, 5 min, 4˚C), and resuspended in freezing medium at a density of 1 to 2 × 10^6 cells/mL.

The cells are aliquoted and frozen in a -80˚C freezer using a freezing container. After 24 hours, they are then transferred to liquid nitrogen.

Maintenance:
Cells should be maintained in suspension at densities from 0.1 to 0.8 × 10^6 cells/mL. Cells are routinely passaged every 2-3 days at the density of 0.1 to 0.2 × 10^6 cells/mL. The cell density is not to exceed 1 × 10^6 cells/mL. For the assay, cells should be seeded in accordance with the following pre-culture condition (See **Preparation of the cells for the assay**, p. 7).

After determination of cell density, the cells are sub-cultured into new flasks with fresh medium, by centrifugation (e.g., approximately 250g, 5 min, 4°C). Alternatively, cells can be also passaged by diluting using fresh medium without counting and centrifugation.

Subculture with counting and centrifugation is to be performed at least once a week.

Cells can be propagated up to two months after thawing but not in excess of 30 passages.

The following table shows a suggested maintenance schedule:

	Day1 (e.g. Fri)	Day 2	Day3	Day4 (e.g. Mon)	Day 5	Day6 (e.g. Wed)	Day 7	Day8 (e.g. Fri)
Maintenance	Subculture 0.2 × 10^6 cells/mL			Subculture 0.2 × 10^6 cells/mL		Subculture 2-fold dilution		Subculture 0.2 × 10^6 cells/mL

Preparation of the cells for the assay:
Before any assay, THP-1 cells are seeded at a density of either 0.1 × 10^6 cells/mL or 0.2 × 10^6 cells/mL, and pre-cultured in culture flasks for 72 hours or for 48 hours, respectively.

Example of a testing schedule

	Fri	Mon	Tue	Wed	Thu	Fri
Exp. 1	**PC**	**E**	**A**			
Exp. 2		**PC**		**E**	**A**	
Exp. 3		**PC**			**E**	**A**

Note:
PC (Pre-culturing, seeding at at a density of either 0.1 × 10^6 cells/mL or 0.2 × 10^6 cells/mL)
E (exposure) / A (analysis)

Pre-test analysis:
Before testing, the quality of each batch of THP-1 cells should be checked and verified as described below. In particular, monitoring of the doubling time of the THP-1 culture and reactivity check must be performed for each batch of thawed cells. High quality maintenance of the flow cytometry is considered essential for reliable data acquisition (See section on **Handling of Equipment**, p.9).

- **Monitoring of the doubling time:**
  In order to obtain reliable results, it is essential that properly cultured cells are used. The doubling time of all new batches of cells should be calculated before starting a test. The doubling time of THP-1 cells has been shown to be approximately 26 hours (data from ATCC), but could fluctuate

depending on the batch and the source. The lead laboratories have shown that the average doubling time, calculated according to the method described below, is approximately 43 hours (range 30-55 hours). Therefore, test facilities should accumulate historical data and set an acceptance range for doubling time.
Measurement of the doubling time: Cells are seeded at the density of 0.2×10^6 cells/mL. Cells are incubated in a 37°C in a 5% CO_2 incubator. The density of the cell suspension is measured at 24, 48, and 72 hours after seeding.
Record the data in a table similar to the one below:

Date of analysis	Time after seeding (hours)	Density of cell suspension (10^6 cells/mL)
yyyy/mm/dd	0	0.2
yyyy/mm/dd	24	
yyyy/mm/dd	48	
yyyy/mm/dd	72	

For each 24 hour period, calculate the doubling time using the following formula (i.e. for the periods from 0 to 24 hours, 24 to 48 hours, and 48 to 72 hours; $conc_{high}$: concentration of cells at time t (24, 48 or 72 hours), $conc_{low}$: concentration of cells at time t-24 hours):

$$\text{Doubling time} = 24 \times \frac{\log_{10}(2)}{\log_{10}(conc_{high}) - \log_{10}(conc_{low})}$$

The final doubling time for the cell culture is the average value of the three calculated consecutive doubling times.

If the cells do not grow properly (i.e., deviate from the acceptable range of the doubling times), or if there is excessive debris or contamination, the following steps must be taken:

1. Use a new batch of frozen cells.
2. Buy new seed cells from a cell bank, if necessary.
3. Confirm CO_2 concentration and other parameters in your incubator.
4. Use new culture medium or other devices. This is especially important if contamination is discovered, in which case all equipment must be cleaned and all materials remade.

- **Reactivity check:**

  The reactivity check should be done two weeks after thawing a new cell batch. Only the cells which passed the reactivity check are to be used for the assay. Test method users should maintain a historical database of data generated with the reactivity checks and with the positive and solvent controls and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time. The reactivity check is performed according to the procedure described below:

  - *Preparation of stock solutions for three control chemicals:*
    DNCB (2,4-dinitrochlorobenzene) and $NiSO_4$ (nickel sulfate) are used as positive control chemicals and LA (lactic acid) as a negative control chemical. First, the three chemicals are weighed and dissolved in the specified solvent. Examples of the procedure are shown below:
    - DNCB: weigh 10 mg in 2 mL volumetric flask and add DMSO up to 2 mL (5mg/mL). Mix and dilute by adding 3 mL of DMSO for a final concentration of 2 mg/mL.
    - $NiSO_4$: weigh 20 mg in 2 mL volumetric flask and add saline up to 2 mL (10 mg/mL).
    - LA: weigh 200 mg in 2mL volumetric flask and add saline up to 2 mL (100 mg/mL).

    Each stock solution should be kept in the dark until the working solution is prepared.
  - *Preparation of working solutions for the three control chemicals:*
    For DNCB, the working solution (8 µg/mL) is prepared by diluting the stock solution 250 times with culture medium. For other chemicals, the working solutions (200 µg/mL of

$NiSO_4$, 2000 µg/mL of LA) are prepared by diluting the stock solution 50 times with culture medium. Medium without chemicals and with only the DMSO solvent (250-fold dilution) are prepared as medium and solvent controls, respectively. For more information, see the text box below.

> The test concentrations of DNCB and $NiSO_4$, which should induce marked up-regulation of both CD86 and CD54 expression and slight cytotoxicity, have been established by the lead laboratories as the commonly used concentrations. These concentrations have been shown to give consistent results (positive outcomes) in other laboratories.
> When DNCB and $NiSO_4$ at these concentrations do not induce CD86 and CD54 expression without affecting cell viability, the CV75 of DNCB and $NiSO_4$ should be re-determined for the specific lot/batch of cells and the newly determined 2 × CV75 concentrations should be used as working solutions (See section on **Dose Finding Assay**, p. 10). The tested concentration (1000 µg/mL) for LA is set by the lead laboratories as the default highest concentration, which does not affect CD86 and CD54 expression and should result in negative outcome.

- *Preparation of cell suspension:*
  Pre-cultured cells are collected by centrifugation (e.g., approximately 250g, 4˚C, 5 minutes) and re-suspended in fresh culture medium at a density of 2×10^6 cells/mL. 500 µL of cell suspensions are added to each well of a 24-well flat-bottom plate.
- *Exposure of the chemicals and flow cytometry analysis:*
  500 µL of working solution are added to the cell suspension in the well (final concentrations of each chemical are 4 µg/mL for DNCB, 100 µg/mL for $NiSO_4$, and 1,000 µg/mL for LA). After incubating for 24 hours, cells are collected, stained, and analyzed using fluorescein-conjugated antibodies as, described in the section on **Measuring CD86/CD54 Exposure** - **Day 2: Staining and Analysis** (p. 15 onwards).
- *Acceptance criteria for the reactivity check*
  - Cell viability of non-treated cells, which are cultured in the medium for 24 hours, should be more than 90%.
  - Both DNCB and $NiSO_4$ should produce a positive response for both CD86 and CD54. (See section on **Prediction Model**, p. 20)
  - LA should produce negative response for both CD86 and CD54. (See section on **Prediction Model**, p. 20)

If the above acceptance criteria are not met for positive control, one of probable cause is that the CV75 values are not appropriate for the cell lot/batch used. In such case the CV75 should be re-determined as described in the section on **Dose Finding Assay** (p.10), and the newly re-calculated CV75 values are to be used to determine the concentrations of control chemicals. The viability is to be below 90% for a tested concentration to be considered appropriate. Please note that you can use two or more concentrations (e.g., 4.0 µg/mL plus CV75) at the same time for the reactivity check and choose one of them to evaluate the reactivity of the cells. In order to consider the cells reactive, the chosen concentration of the chemicals is to meet the acceptance criteria of the reactivity check (See above). Even if DNCB or $NiSO_4$ is false negative, or LA is false positive in the first run, the cells can be used when the acceptance criteria are met in both the second and third runs. If not, a new batch of cells is to be prepared. Furthermore, if LA is systematically positive, FL-1 voltage should be checked and optimized to meet the acceptance criteria for this chemical. (See section on **Measuring CD86/CD54 Exposure - Day 2: Staining and Analysis** - *Flow Cytometry Acquisition*, p. 16).

HANDLING OF EQUIPMENT

The basic calibration of flow cytometer is to be done with appropriate calibration beads following the manufacturer's instructions. The flow cytometer has to be set before testing. It is not necessary to modify the setting before each run if the mean fluorescence intensity of control cells stained by mouse IgG has

not changed. The outline of the settings is shown in the sections on **Measuring CD86/CD54 Exposure** - **Day 2: Staining and Analysis -** *Flow Cytometry Acquisition* p.16.

Test Material Exposure Procedures

DOSE FINDING ASSAY (PI ASSAY)

DAY 1: PREPARATION OF CHEMICALS AND TREATMENTS

- *Solvent selection:*
  Solubility of each chemical is evaluated and confirmed visually. An appropriate solvent will dissolve the test chemical completely, i.e. the solution must not be cloudy or have noticeable precipitate. The following solubilization procedure for the selection of the appropriate solvent is to be followed:
  - First, try to dissolve the chemical in saline. For example, add up to 1 mL of saline to 0.1 g of the test chemical in a volumetric flask. RPMI 1640 can be used as an alternative to saline, if solubility is compatible between saline and RPMI 1640.
  - If the chemical is soluble in saline at 100 mg/mL, saline is to be used as the solvent.
  - If not, the chemical should be dissolved in DMSO at 500 mg/mL. Add up to 1 mL of DMSO to 0.5 g of the test chemical in a volumetric flask.
  - If the chemical is not soluble at 500 mg/mL, the highest soluble concentration (HSC) should be determined by diluting the solution from 500 mg/mL in a common ratio of two (250 mg/mL → 125 mg/mL → continue if needed). Minimum concentration is set to 1 mg/mL.
  - Saline is to be used as the solvent for testing surfactant agents. If a surfactant agent is not soluble in saline at 100 mg/mL, the highest soluble concentration in saline is determined by diluting the solution from 100 mg/mL in a common ratio of two (50 mg/mL → 25 mg/mL → continue if needed). Minimum concentration is set to 1 mg/mL.

Note :

- If the test chemical is not soluble, try 5 minutes of sonication.
- Solvents other than saline and DMSO may be used if sufficient scientific rationale is provided.

- *Preparation of solutions and exposure to THP-1 cells:*

1. *Stock solution:*
   Stock solutions (8 doses) are prepared by 1:2 serial dilutions from 100 mg/mL in saline, 500 mg/mL in DMSO, or the highest soluble concentration in either saline or DMSO (i.e. saline for chemicals that dissolve in saline, DMSO for chemicals that dissolve in DMSO).
2. *Working solution:*
   For working solutions that use saline as a solvent, dilute each stock solution 50 times with culture medium (e.g., 50 µL of stock solution in 2.45 mL of culture medium). For working solutions that use DMSO as a solvent, dilute each stock solution 250 times with culture medium (e.g., 10 µL of stock solution in 2.49 mL of culture medium). Working solutions include stable suspensions. For working solutions of lipophilic chemicals, sonication is recommended to ensure uniform distribution of the test chemical in the medium. The sonication is to last no more than 5 minutes in duration.
3. *Cell suspension:*
   Cell suspensions are prepared by centrifugation (e.g.: approximately 250g, 4˚C, 5 min) of culture flasks and re-suspension in fresh culture medium at a density of 2×10^6 cells/mL.
   Add 500 µL of cell suspension to each well (1×10^6 cells/well) of 24-well flat-bottom plates.
   Add 80 µL of cell suspension to each well (1.6×10^5 cells/well) of 96-well flat-bottom plates.
4. *Exposure:*
   Equal volumes of each working solution are added to the cells:
   Add 500 µL of working solution for 24-well flat-bottom plates.
   Add 80 µL of working solution for 96-well flat-bottom plates.

Working solutions are to be added drop-wise, and the plate is to be shaken by hand before being placed in the incubator. Cells at a final density of 1.0 × 10^6 cells/mL are cultured for 24 hours in a 5% CO_2 incubator with 8 concentrations of each test chemical.

Examples of plate layouts

For 24 well plate:

1. Medium	2. DMSO	3. Sample A HSC/2^7	4. HSC/2^6	5. HSC/2^5	6. HSC/2^4
7. Sample A HSC/2^3	8. HSC/2^2	9. HSC/2	10. HSC		
11. Sample B HSC/2^7	12. HSC/2^6	13. HSC/2^5	14. HSC/2^4	15. HSC/2^3	16. HSC/2^2
17. Sample B HSC/2	18. HSC				

HSC: highest soluble concentration

For 96 well plate:

	1	2	3	4	5	6	7	8	9	10	
	11	12	13	14	15	16	17	18			

Each number corresponds to the number shown in the upper figure (24 well plate format)

Note:

- Chemicals are to be kept in the dark as much as possible.
- All steps from preparation of chemicals to exposure are to be performed within one hour in order to minimize degradation or breakdown of any chemical, especially unstable ones, like hydroquinone.
- PI-assay yield identical data for both 24-well and 96-well formats.
- It is recommended that no more than three chemicals per run be tested and each chemical should be tested in at least two independent runs to derive a final prediction.

DAY 2: STAINING, ANALYSIS AND CALCULATION OF CV75

Note:

- The FACS buffer should be prepared day before and the blocking solution just before use.
- For staining and analysis, all solutions are to be kept at between 2 and 8°C. During the procedure, the cells are to be kept on ice and in the dark as much as possible.

- *Collection of cells:*

1. Exposure in 24-well flat-bottom plate: Cells are transferred into sample tubes and collected by centrifugation (approximately 250g, 5 minutes, 4˚C). Supernatants are discarded and remaining cells are re-suspended in 600 µL of FACS buffer (PBS with 0.1% BSA). 200 µL of suspension is transferred into a 96-well round-bottom or V bottom plate.
2. Exposure in 96-well flat-bottom plate: All cell suspensions are transferred into a 96-well round-bottom plate.

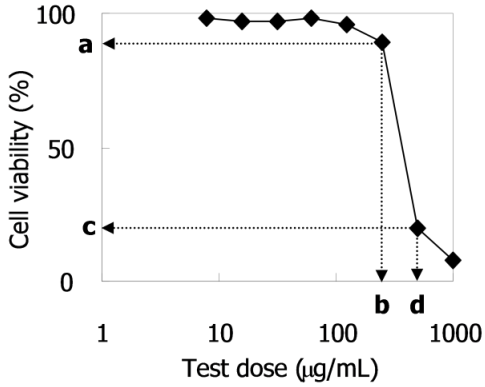
- *Cell staining with propidium iodide (PI):*
  The cells are washed twice with 200 µL of FACS buffer and re-suspended in 200 µL of FACS buffer. 10 µL of PI solution (12.5 µg/mL diluted in PBS) is added just before FACS analysis (final concentration of PI = 0.625 µg/mL). If FACS analysis is performed with 5 mL tubes, cell suspensions are transferred to the 5 mL tube after washing, after which 400 µL of FACS buffer and 20 µL of PI solution are added.

- *Flow cytometry analysis:*
  Cell viability is measured by gating-out dead cells stained with PI. A total of 10,000 living cells is acquired from sample tubes (For 96-well plates, 10,000 living cells are also acquired). When cell viability is low, acquire up to 30,000 cells, including dead ones. Alternatively, the acquisition can be finished 1 minute after starting. The cell viability is shown by the cytometry analysis program (e.g. % total), or can be calculated using the following formula:

$$\textbf{Cell Viability} = \frac{\textbf{Number of living cells}}{\textbf{Total Number of acquired cells}} \textbf{*100}$$

Details on flow cytometry settings can be found in the section **Measuring CD86/CD54 Exposure** - **Day 2: Staining and Analysis -** *Flow Cytometry Acquisition* (p.16). An example of flow cytometry analysis is shown in ***Annex 1*** (p.21).

- *Estimation of CV75 value:*
  The % of living cells (PI-negative cells) is used as the value for cell viability.



Example of the result of PI assay

The CV75 value is derived from the dose response curve as shown in the figure (75% of cell viability, lying between **a** and **c**). CV75 is defined as the estimated concentration that is required to elicit 75% cell viability. The CV75 value is calculated by log-linear interpolation utilizing the following equation:

$$\textbf{Log CV75} = \frac{\textbf{(75 – c) × Log b – (75 – a) × Log d}}{\textbf{a – c}}$$

Note:

- At least two independent PI assays should be performed to derive a reliable CV75 for each chemical. The mean of at least two CV75 values is used for setting the dose-range for measuring CD86 and CD54 expression.
- When cell viability is less than 75% at the lowest dose, the CV75 cannot be calculated. In which case, the PI assay is to be performed again after lowering the highest concentration of stock solution.
- If the default highest concentration (either in saline, DMSO, or other solvents) is non-toxic, the highest concentration should be re-determined. It is recommended in any case not to exceed the final concentration in the plate of 5,000 µg/mL for chemicals solubilised in saline or 1,000 µg/mL for chemicals solubilised in DMSO.
    - The highest soluble concentration for chemicals solubilised in saline is to be determined without exceeding a final concentration of 5,000 µg/mL (500 mg/mL of stock solution).
    - The highest soluble concentration for the chemicals solubilised in DMSO could be reconfirmed by more detailed manner. For instance, if highest soluble concentration is 250 mg/mL stock solution but the result is non-toxic, highest soluble concentration could be reconfirmed between 250 mg/mL and 500 mg/mL.
    - See section on *"Calculating the test doses"* below for more information on chemicals for which still no CV75 can be determined.
- When the range between the doses lying directly above and below the CV75 value is large (e.g. more than 500 µg/mL), or when cell viability drops sharply in a dose dependent manner, stock solutions are to be prepared by using a narrower dilution factor than 1:2 order to obtain a more accurate determination of the CV75 value.
- Measurement of CD86/54 expression is to be conducted under conditions approximating the dose finding assay. Therefore, the CD86/54 expression should be measured shortly after determining CV75, using the same batch of THP-1 cells.

- *Calculating the test doses:*
    - Eight final concentrations (µg/mL) are used for each chemical tested (See section on **Measuring CD86/CD54 Expression** below). These are at 1.2, 1, 1/1.2, $1/1.2^2$, $1/1.2^3$, $1/1.2^4$, $1/1.2^5$, and $1/1.2^6$ × CV75.
    - If CV75 cannot be determined, stock solution (8 doses) will be prepared by serial 1:1.2 dilutions from the highest soluble dose. The final maximum concentration of test chemicals is not to exceed **5,000 µg/mL for saline or 1,000 µg/mL for DMSO**

Endpoint Measurement

MEASURING CD86/CD54 EXPRESSION

DAY 1: CHEMICAL EXPOSURE

- *Stock solutions:*
  For each chemical, the highest dose solution is prepared first:
    - If the chemical is soluble in culture medium or saline, the solution is to be prepared so that the final concentration in the plate is 100 times the concentration corresponding to 1.2 × CV75. Weigh the test chemical in a volumetric flask and add the solvent up to the given volume (For example, if CV75 value is 50 µg/mL, 1.2 × CV75 value is 60 µg/mL. Therefore 6 mg of test chemical is weighed in a volumetric flask and then add up to 1 mL of culture medium or saline).
    - If the chemical is soluble in DMSO, the solution is prepared so that the final concentration in

the plate is 500 times the concentration corresponding to 1.2 × CV75. This solution should be made as described above. If CV75 is not determined, the highest soluble concentration should be used as the starting dose.

- Note: If 1.2 × CV75 is lower than 10 µg/mL, a ten times higher concentrated stock solution should be prepared in order to be able to accurately weigh out the chemical. Once the ten times higher concentration solution is prepared, it is then diluted ten times with a suitable solvent for preparation of the highest dose stock solution.
- The other 7 stock solutions are prepared by serial 1:1.2 dilution, by transferring 500 µL to a vial or tube containing 100 µL of solvent. The stock solutions have to be prepared freshly before each assay.

- *Working solutions:*
  The working solutions are prepared by diluting each stock solution in culture medium.
  - If the chemical is solubilised in culture medium or saline, the stock solutions are diluted 1:50 (e.g., 50 µL of stock solution in 2450 µL of culture medium)
  - If the chemical is solubilised in DMSO, the stock solutions are diluted 1:250 (e.g., 10 µL of stock solution in 2490 µL of culture medium).
  - If precipitates are observed, sonication is performed to ensure uniform distribution in the medium. The sonication is to last no more than 5 minutes in duration.

- *Cell suspension:*
  Cells are collected from culture flasks by centrifugation (approximately 250g, 4˚C, 5 minutes) and then re-suspended in fresh culture medium at a density of 2 × 10^6 cells/mL. Cells are distributed into a 24 well flat-bottom plate with 500 µL (1 × 10^6 cells/well).

- *Exposure:*
  Equal volumes (500 µL) of working solution are added to the cells, which are then cultured for 24±0.5 hours. Each final concentration of DMSO or saline in test chemical exposure is 0.2% or 1%, respectively. A medium control sample and a solvent control sample for DMSO (final concentration; 0.2%) should be included. A solvent control sample for saline is not needed because medium control can be its alternative.
  Additionally, DNCB should be tested as the positive control in each assay, at a final concentration of 4.0 µg/mL, yielding approximately 70-90% of cell viability. Alternatively, the CV75 of DNCB, which is set by each test facility, could be also used as the positive control dose based on the cytotoxicity data. Two or more concentrations (e.g., 4.0 µg/mL plus CV75) are allowed to be also tested at the same time.

- *Replication:*
  In each run, a single replicate for each concentration of the test chemical and control substance is sufficient because a prediction is obtained from at least two independent runs.

An example template is shown below (24 well plate):

1. Medium	2. DMSO	3. DNCB 4µg/mL	4. sampleA CV75/1.2^6	5. CV75/1.2^5	6. CV75/1.2^4
7. SampleA CV75/1.2^3	8. CV75/1.2^2	9. CV75/1.2	10. CV75	11. CV75*1.2	12. Medium Or DMSO
13. sampleB CV75/1.2^6	14. CV75/1.2^5	15. CV75/1.2^4	16. CV75/1.2^3	17. CV75/1.2^2	18. CV75/1.2
19. sampleB CV75	20. CV75*1.2				

Note:

- Positive controls are used to demonstrate appropriate performance of the assay and competency of the test facility to successfully conduct the assay. The positive control should produce a positive response in both CD86 and CD54.
- If more than one chemical is tested at the same time, a solvent control is set on each test group of chemicals.
- When planning the experiments, consider that each chemical should be tested in at least two independent runs
- More than one run for a chemical is allowed to be performed on the same day, provided that each run meets the following conditions:
    1. Cells are collected from different culture flasks.
    2. Working solutions of the chemical and antibody solutions are prepared separately for each run.

DAY 2: STAINING AND ANALYSIS

- *Collecting the cells and FcR blocking:*

Note:

- The FACS buffer should be prepared a day before and the blocking solution just before use.
- For the staining and analysis step, all solutions must be kept at the temperature of approximately 2 to 8°C. During the procedure, the cells must be kept as much as possible on ice and in the dark.

Chemically-treated cells are transferred to sample tubes, collected by centrifugation (approximately 250g, 5 min, 4˚C) and then washed twice with 1 mL of FACS buffer. If necessary, additional washing steps may be done. After the wash, cells are blocked with 600 µL of blocking solution (staining buffer containing 0.01% (w/v) globulin (Cohn fraction II, III, Human: SIGMA, #G2388-10G)) at 4˚C for 15 min. After blocking, cells are split in three aliquots of 180 µL (approximately 0.3×10^6 cells/well) in 96 well plates (round or V bottom wells) before staining with antibodies.

Note:
FcR blocking can be performed before or following cell division. The Test Developers have found that there is no difference either way.

- *Cell staining with FITC-labelled anti-CD86, CD54 antibody and mouse IgG1:*
  The staining concentration of each antibody is shown in the table below:

	Volume of antibody	The # of cells	Total volume of working solution /sample
Anti-CD86 antibody	6 µL	3×10^5	50 µL
Anti-CD54 antibody	3 µL	3×10^5	50 µL
FITC labelled-mouse IgG1	3 µL	3×10^5	50 µL

Pre-mixed antibody solutions are prepared. Either 6 µL or 3 µL of antibody, depending on the antibody used, is added to 44 µL or 47 µL of FACS buffer to prepare 50µL of pre-mixed solution per sample. A master mix should be prepared based on the number of samples stained with the same antibody (e.g. if the sample number is 20, 1000 µL pre-mixed solution of each antibody is needed. A 1100 uL master mix should be prepared, and, for CD86, 132 uL of antibody and 968 uL of FACS buffer are mixed).

The three groups of cells are centrifuged and the entire 50 µL of pre-mixed antibody solution is added to each cell pellet. After gently mixing by hand, cells are incubated at 4˚C for 30 min in the dark.

- *Preparation of samples for measurement:*
  After staining with antibodies, the cells are washed twice with 150 µL or more of FACS buffer (Buffer should be removed from the cell pellet very carefully to avoid contamination of antibodies or cells between samples) and re-suspended in a final volume of 400 µL/tube (for acquisition in sample tubes), 200 µL/tube (for acquisition in 2 mL tubes) or 200 µL/well (for acquisition with an automated 96-well plate sampler) of FACS buffer. Before flow cytometry acquisition, 13.125 µg/mL PI solution is added to obtain the final concentration of 0.625 µg/mL, i.e. 20 µL of PI solution in each when using 5 mL tubes, or 10 µL of PI solution in each when using 2 mL tubes or 96-well plates.

- *Flow Cytometry Acquisition:*
  Expression of cell surface antigens is analyzed by flow cytometry. The FITC acquisition channel (FL-1) should be set for the optimal detection of the FITC fluorescence signal, and the PI acquisition channel (FL-3) should be set for the optimal detection of DNCB-bound PI fluorescence signal.
  The Excitation and Emission wavelength of FACSCalibur (Beckton Dickinson) are shown below, for example:
  - Excitation wavelength: **488 nm** (Argon laser)
  - Emission wavelength: **FL-1 530 ± 15 nm / FL-3 > 650 nm**

  Preparation of the acquisition:

  - The following acquisition plots are prepared:
    i. 2D plot consisting of FSC (Forward Scatter) vs SSC (Side Scatter)
    ii. 2D dot plot consisting of FL-1 vs FL-3
    iii. Histogram plot of each channel (FL-1 and FL-3, respectively)
  - Medium-treated THP-1 cells and DNCB-treated THP-1 cells are prepared and stained by FITC-conjugated anti-CD86 antibody, FITC-conjugated mouse IgG (“isotype”), and propidium iodide, respectively, according to the procedure for acquisition.
  - Set the voltage of FSC and SSC to appropriate levels. FSC and SSC are not needed for the analysis, but the FSC/SSC plot should be checked to make sure that a single population appears without contamination or excessive debris.
  - Set and compensate the FL-1 and FL-3 voltage to appropriate position. Please note that, in some machines (ex. FACSCanto), reliability and reproducibility of quantitative data can be assured by setting the FL-1 voltage to the optimal voltage in order to minimize the noise signal effect for low fluorescence levels.
  - The FL-1 voltage is set using the FITC labeled-mouse IgG1-treated cells, such that:
    - FACSCalibur (Becton Dickinson): The Mean Fluorescence Intensity (MFI) of control cells is set in the range between approximately 3.0 and 4.0.
    - FACSCanto (Becton Dickinson): The Mean Fluorescence Intensity (MFI) of control cells is set in the range of approximately 1000 to avoid the effect of noise at low voltages in the FACSCanto
    - EPICS XL-MCL System II (Beckman Coulter): The MFI of control cells is set in the range between approximately 0.4 and 0.5.
  - Set R1 gate at the middle position between the peak of the negative fraction and the positive fraction in the FL-3 histogram using DNCB-treated cells. The negative fraction corresponds to the living cells and is kept for the subsequent analyses. The percentage of R1-gated cells should exceed 95%.
  - Check for leaks of fluorescence following the manufacturer's instructions. If necessary, the compensation of FL-1 and FL-3 voltage should be conducted using FITC labeled-CD86 antibodies and PI stained DNCB-treated cells.
  - Attention should be paid to the maintenance of the cytometry in accordance with the manufacturer’s instructions. In particular, the process of washing should be conducted very carefully because insoluble chemicals could flow in the flow line.

Acquisition:

- Dead cells are gated-out by staining with PI. Gating by FSC (forward scatter) and SSC

(side scatter) is not done. A total of 10,000 living cells are analysed. If cell viability is low, up to 30,000 cells including dead cells should be acquired. Alternatively, the data acquisition can be finished 1 minute after starting of the acquisition.

- Mean fluorescence intensity (MFI) of viable cells and viability for each sample are used to analysis.
- When the cell viability is less than 50%, the relative fluorescence intensity (RFI) is not used because of the diffuse labelling cytoplasmic structures that are generated following cell membrane destruction.

An example of flow cytometry analysis is shown in **Annex 2** (p.22)

Acceptance Criteria

REQUIREMENT FOR QUALIFIED TESTING

- Cell viability of medium and DMSO controls should be more than 90%.
- In the positive control (DNCB), RFI values of both CD86 and CD54 should be over the positive criteria (CD86 ≥ 150 and CD54 ≥ 200) and cell viability should be more than 50%.
- In the DMSO solvent control, RFI values compared to the medium control of both CD86 and CD54 should not exceed the positive criteria (CD86 > 150 and CD54 > 200).
- For both medium and DMSO controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%.

If these requirements are not satisfied, the run is discarded. In that case, monitor the state of the cells (doubling time, shape, passage number, etc.) as described in the section on **Routine Culture Procedure → Pre-test analysis** (p.7).

ABNORMAL VALUES

- RFI values cannot be less than zero. Regardless of the reason (e.g., clog of flow line), such values should be omitted from the prediction.
- If an abnormal value (for instance, strongly induced CD86 or CD54 expression at only one non-cytotoxic concentration) is observed, check whether there are abnormal conditions in the run and record them in the reporting section.

REQUIREMENT FOR DATA ACCEPTANCE

When following requirements are not satisfied, the results for this chemical are discarded:

- For the test chemical resulting in negative outcome, the cell viability at the 1.2 × CV75 should be less than 90%.

> *Note 1:*
> If, for the test chemical resulting in positive outcome, the cell viability at the 1.2 × CV75 is more than 90%, the data is acceptable.
>
> *Note 2*:
> When 5,000 µg/mL in saline, 1,000 µg/mL in DMSO, or highest soluble dose is used as the maximal test concentration instead of CV75-based dose, the data for test chemical are accepted regardless of the cell viability.

- Cell viability of at least 4 doses in each assay should be > 50%.

In these cases, more appropriate doses should be set by repeating the CV75 determination assay.

Data Analysis

- *Flow cytometry analysis:*

The Relative Fluorescence Intensity (RFI) is used as an indicator of CD86 and CD54 expression, and is calculated as follows for each concentration of every chemical:

$$\text{RFI} = \frac{\text{MFI of chemical treated cells- MFI of chemical -treated isotype cells}}{\text{MFI of solvent treated cells- MFI of solvent -treated isotype cells}} \times 100$$

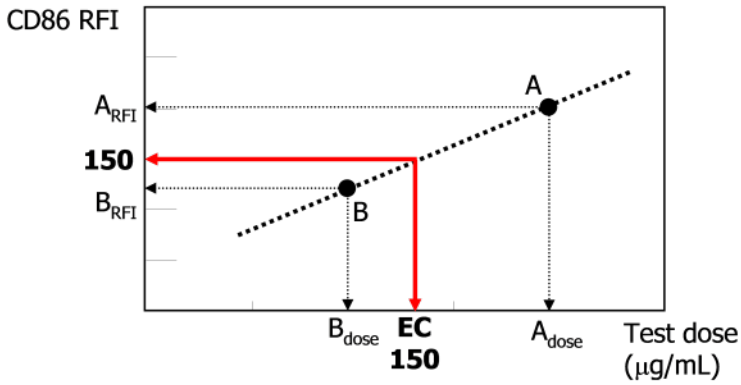
MFI = Geometric mean fluorescence intensity

For each concentration of every chemical, the cell viability is recorded from the isotype control cells (stained with FITC labelled-mouse IgG1).

> *Note:*
> A chemical with its own intrinsic fluorescence may influence the MFI values recorded for the cells treated with it. However, it is not necessary to adjust the MFI values in this case because the formula described above can correct such effects.

- *Calculation of EC150 and EC200*

For the test chemicals considered to be sensitisers, two effective concentrations (EC) values, the EC150 for CD86 and EC200 for CD54, (i.e. the concentration at which the test chemicals-induced a RFI of 150 or 200) can be calculated.



If,

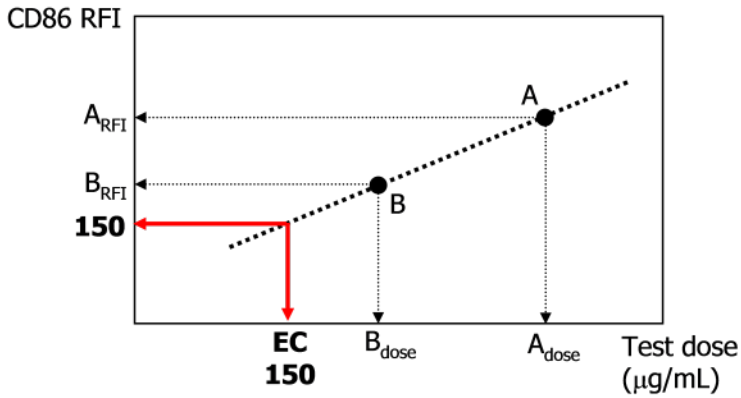
- Higher dose is pointed as A (A_{dose} , A_{RFI}),
- Lower dose is pointed as B (B_{dose} , B_{RFI}), and
- Positive criteria (RFI = 150 for CD86 and RFI = 200 for CD54) lie between A_{RFI} and B_{RFI} ,

Then:

EC150 and EC200 would be calculated by linear interpolation as follow:

$$EC150\ (\text{for CD86}) = B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$
$$EC200\ (\text{for CD54}) = B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

In the case that the RFI value at the lowest dose is above the positive criteria, EC150 (CD86) or EC200 (CD54) is calculated using the lowest dose and the second lowest dose by log-linear extrapolation, according to the formula below.



EC150 (for CD86) = 2^ {Log2 (B_{dose}) + (150 − B_{RFI}) / (A_{RFI} − B_{RFI}) × [Log2(A_{dose}) – Log2(B_{dose})]}
EC200 (for CD54) = 2^ {Log2 (B_{dose}) + (200 − B_{RFI}) / (A_{RFI} − B_{RFI}) × [Log2 (A_{dose}) – Log2(B_{dose})]}

Note:
For any case in which the RFI value at the second-lowest dose was less than 10% more than that at the lowest dose, the third- or fourth-lowest dose (i.e., 1.2^2- or 1.2^3-fold higher (respectively) than the lowest dose) was used to calculate the EC value if the RFI value at the third- or fourth-lowest dose was > 10% more than that at the lowest dose.
See the example below: here, the third-lowest dose (lower dose) was selected when both the third- and fourth-lowest doses met the criteria.

Sample	Dose (μg/mL)	Log2(conc.)	CD86 RFI Case 1	CD86 RFI Case 2
Chemical A	85.3	6.4	184	174
	102.4	6.7	192	179
	122.9	6.9	232	182
	147.5	7.2	264	228
	176.9	7.5	337	284
	212.3	7.7	369	273
	254.8	8.0	461	223
	305.8	8.3	328	150

The difference between two RFI values is less than 10%.

The difference is more than 10% by using third lowest dose (1.2^2-fold higher than lowest dose).

OK

Dose (μg/mL)	Log2(conc.)	CD86 RFI Case 1
85.3	6.4	184
102.4	6.7	192
122.9	6.9	232
147.5	7.2	264
176.9	7.5	337
212.3	7.7	369
254.8	8.0	461
305.8	8.3	328

The difference is more than 10% by using fourth lowest dose (1.2^3-fold higher than lowest dose).

OK

Dose (μg/mL)	Log2(conc.)	CD86 RFI Case 2
85.3	6.4	174
102.4	6.7	179
122.9	6.9	182
147.5	7.2	228
176.9	7.5	284
212.3	7.7	273
254.8	8.0	223
305.8	8.3	150

In the h-CLAT at least two independent runs need to be performed to derive a final prediction (see section on **Prediction Model** below) For the purpose of more precisely deriving the EC150 and EC200 values, three independent runs should be performed. The EC150 and EC200 values to be reported are the median values derived from three independent positive runs. When only two of three independent runs meet the positive criteria, the highest EC150 or EC200 value is adopted. Proposals on the use of these values within integrated approaches for the assessment of sensitising potency, are published in the literature (Nukada et al., 2012, 2013 and Tsujita-Inoue et al., 2014).

Prediction Model

Each chemical is tested in three at least two independent runs (See the sections on **Measuring CD86/CD54 Exposure** (p.13) and on **Data Analysis** (p.18).

If the RFI of CD86 is equal to or greater than 150% at any tested dose (> 50% of cell viability) in at least 2 independent runs, AND/OR if the RFI of CD54 is equal to or greater than 200% at any tested dose (> 50% of cell viability) in at least 2 independent runs, the chemical prediction is considered as positive. Otherwise it is considered as a negative. In case the first two independent runs are not concordant a third run needs to be performed and the final prediction will be based on the mode of the conclusions from the three individual runs (i.e. 2 out of 3).

Note:
When the chemical is tested at 5000 µg/mL in saline, 1000 µg/mL in DMSO, or highest soluble dose as the maximal test concentration instead of CV75-based dose and does not meet the positive criteria above without affecting cytotoxicity at all tested doses, the chemical prediction should be considered as negative.

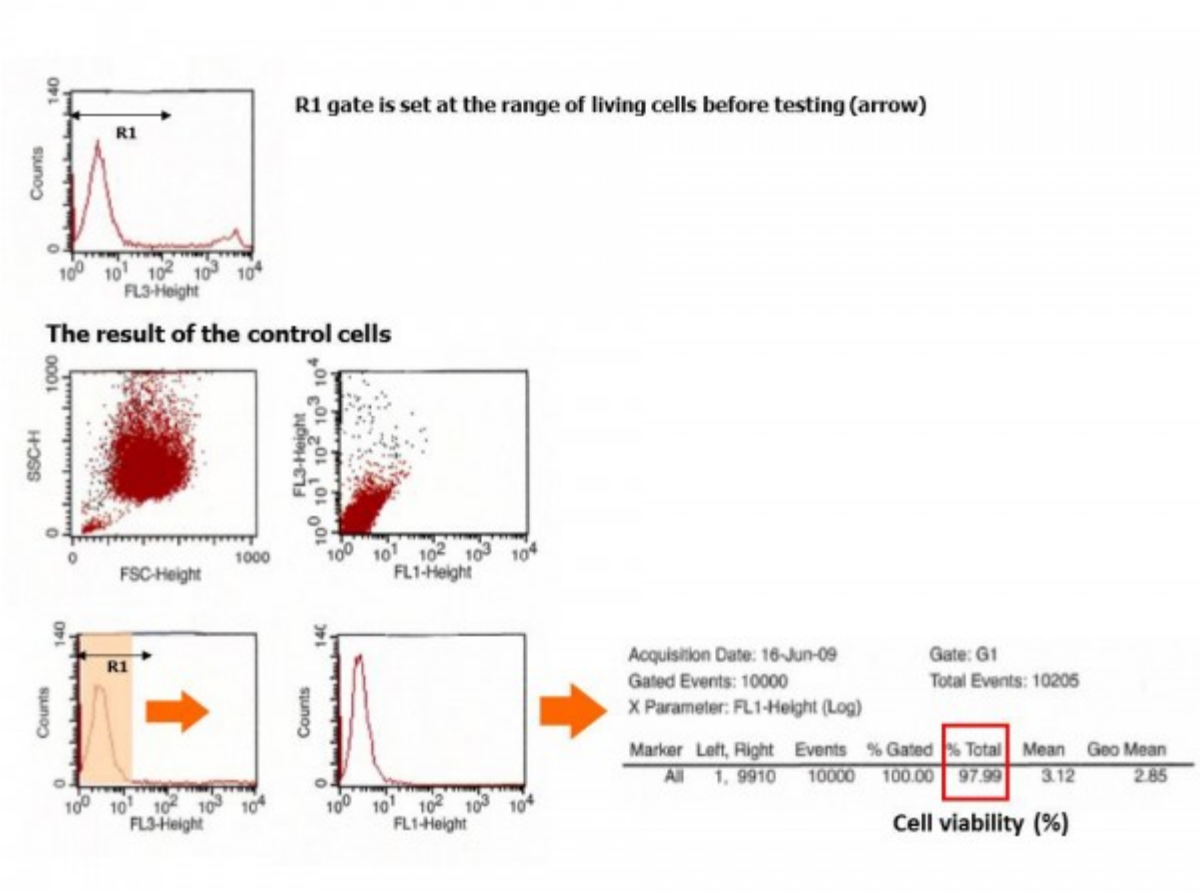
Test chemicals with a Log Kow of up to 3.5 have been tested successfully (Takenouchi et al., 2013). However, test chemicals with a Log Kow of greater than 3.5 may still be tested at lower soluble concentrations. In such a case, a negative result should not be considered, whereas a positive result could still be used to support the identification of the test chemical as a skin sensitiser.

In the light of the above, negative results should be interpreted in the context of the stated limitations and together with other information sources within the framework of IATA.

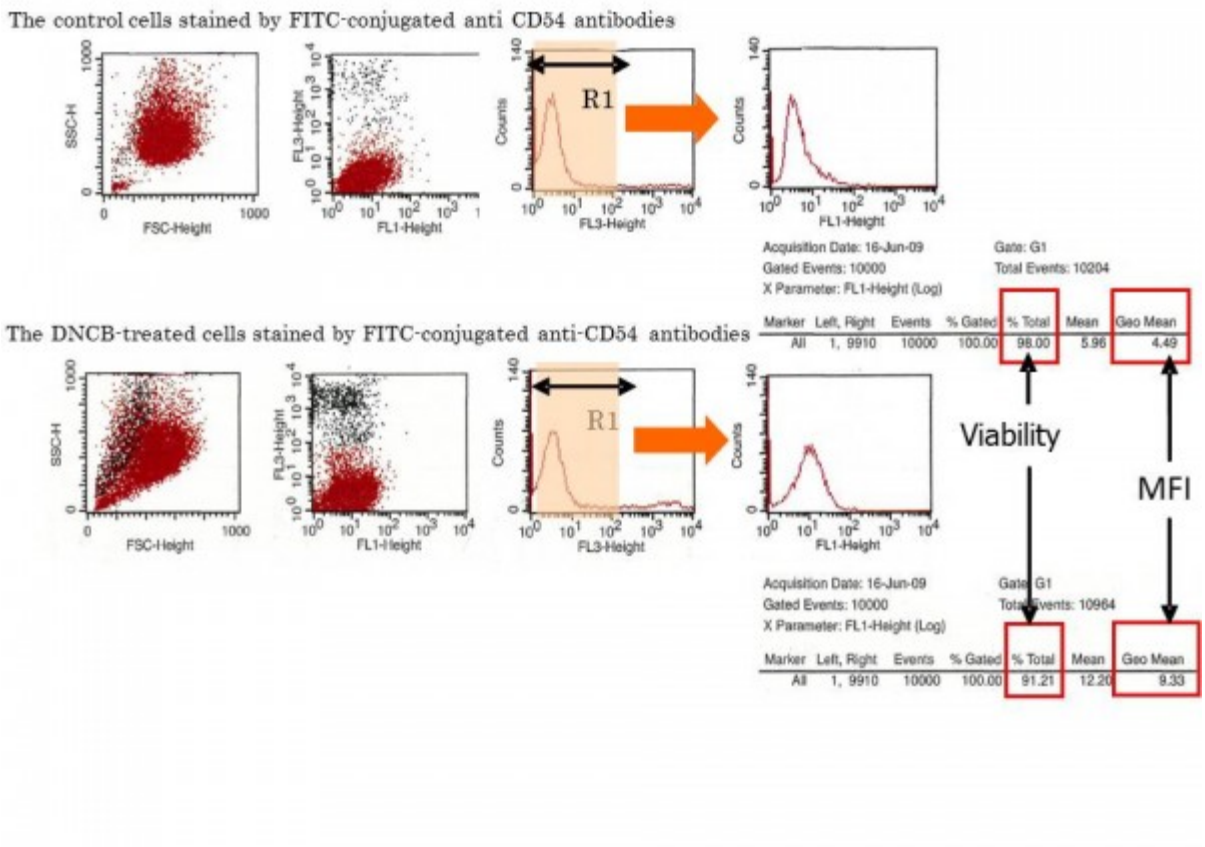
Up to six runs, meeting the requirements for qualified testing, are permitted to reach a conclusion for each chemical. The six runs may include runs for which the data adoption criteria are not met for this chemical (for example, 1: invalid, 2: valid, 3: invalid, 4: invalid, 5: valid, and 6: invalid). If no prediction can be made after the sixth run, the result is inconclusive and the chemical is to be classified accordingly.

Annexes

Annex 1: Example FACS plot in dose finding assay



Annex 2 : Example FACS plot in the measurement of CD86/CD54 expression



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